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Pollen Tetrads in the Detection of Environmental Mutagenesis

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Although pollen viability promises to be a very sensitive indicator of environmental mutagenesis, its utility in this regard is confounded by the fact that it is influenced also by nonmutagenic environmental stress. However, with appropriately homozygous material, we may discriminate between mutagenic and nonmutagenic influences on pollen viability. Pollen inviability resulting from mutagenesis will exhibit a strong tendency to segregate, whereas stress induced inviability will not. When pollen grains are shed individually, evidence for genetic segregation is often lost, but with pollen in tetrads, this evidence, a specific indicator of environmentally induced mutation, is preserved. A further advantage of pollen in tetrads is that, again because evidence for genetic segregation is preserved, tetrads allow us to distinguish between pre- and postpachytene mutations. This capability eliminates the problem of mutant sectors whereby a single mutational event may give rise to a large number of mutant cells. Methods of examining pollen tetrads are discussed.

Pollen exhibits several characteristics which allow it to serve as an exceptionally fine indicator of environmental mutagenesis. It is haploid, available in very large numbers, and microscopic. Furthermore, its viability may be tested by a wide variety of methods and, perhaps most importantly, this viability is dependant upon a large number of loci. Pfahler (*1*) has summarized a series of studies which indicate, not only that even very small deletions in virtually any part of the genome will induce pollen abortion, but also that many point mutations will have equally lethal effects upon the male gametophyte. This suggests that if we employ pollen viability as an indicator of environmentally induced mutation, we shall have a system of exceptional sensitivity, a system in which not one, but perhaps thousands of loci in each pollen grain will serve as possible indicators of genetic changes. What remains to be done now is to determine how the system may be employed, what controls it provides, and what methods and taxa are most promising.

The primary difficulty of working with pollen is that not only is is exceptionally sensitive to genetic changes in the gametophytic genotype, it is also very sensitive to nonmutagenic environmental stresses. For example, once the meiotic process starts, even moderate temperature or drought stress can totally disrupt pollen development. As a consequence of this, pollen viability exhibits an extremely high degree of day to day variation, a fact which could effectively exclude pollen viability as an indicator of environmental mutagenesis were it not for the existence of a suitable control, pollen in tetrads.

In the typical pattern of pollen development (*2*), the callose wall which joins the four haploid products of meiosis breaks down and the individual microspores separate before completing their conversion to mature pollen grains. However, Erdtman (*3*) has reported that, in at least 41 families of flowering plants, there are exceptions to this pattern. In these exceptional cases, microspores do not separate after meiosis. Instead they remain bound to each other as dyads or, more commonly, as tetrads. [Barber (*4*) has discussed the fact that further exceptions are found in the Orchidaceae, the Asclepiadaceae, and the Mimosaceae, exceptions in which the mature pollen grains are shed not singly, and not in dyads or tetrads, but rather in pollinia consisting of hundreds of pollen grains. These have little relevance for the present discussion, however.]

The utility of pollen in tetrads is that each tetrad

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contains the segregational products of a single meiocyte. Thus, if a character which is visually expressed in the pollen is segregating within a meiocyte, the presence of this character should be readily apparent among the pollen grains of the tetrad arising from that meiocyte. Pollen viability, as is explained below, is just such a visibly expressed character. At its point, it is useful to recall that environmentally induced mutations often exhibit two particular characteristics: they tend to cause the death of pollen grains which carry them and they are usually heterozygous, thus segregating. Accordingly, environmentally induced mutations will result in tetrads which are segregating for viable and inviable pollen grains. If the target cell has been a postpachytene cell, a single induced mutation will result in a tetrad which contains only three rather than four viable pollen grains. If, instead, the target cell has been prepachytene, the result will be one or more tetrads which contain two viable pollen grains and two inviable pollen grains. Thus, as long as environmentally induced mutations are sufficiently rare that few meiocytes sustain more than one of them, such mutation will generally result in genetic segregation. In this respect, mutation differs from environmental stresses such as drought or extremes of temperature. These latter stresses are well known sources of pollen inviability but, within highly inbred lines, the inviability that they induce will generally not be characterized by patterns of segregation. [Not all pollen grains within a flower or even anther will be equally influenced by environmental stresses, probably because not all stages of development are equally sensitive to stress and even within an anther, development is far from synchronous (*5*). Furthermore, because environmental stresses very often induce abortion of 100% of the pollen grains, there is also a quantitative difference between the effects of environmental stress and mutagenesis as sources of pollen inviability.] If pollen grains separate at maturity, it will not be possible to distinguish between these two sources of pollen inviability. If, however, pollen is shed in tetrads, the segregation which characterizes the effects of environmental mutagenesis will be immediately apparent. This is the concept upon which the method suggested in this paper is based. It should be emphasized that this concept will be applicable only when the test organism is highly inbred or known to be free of heterozygous temperature or other stress sensitive alleles. Otherwise also environmental stress will result in patterns of segregation.

Table 1. Probabilities of obtaining tetrads with fewer than four viable pollen grains.

Type of tetrad	Probability that this tetrad type will be produced by mutagenic processes[a]
3 viable grains (1 postpachytene mutation)	Probability per microspore = gm Probability per tetrad = $4gm$
2viable grains (1 prepachytene mutation) (2 postpachytene mutations)	Probability per meiocyte = $2gm$ Probability = $4gm \times 3gm$ = negligible
1 viable grain (1 prepachytene mutation plus 1 postpachytene mutation among remaining 2 microspores)	Probability = negligible $2\ gm \times 2gm$
(3 postpachytene mutations)	Probability = $4gm \times 3gm$ = neglibible

[a] g = the number of genes which influence pollen viability; m = the mutation rate per gene.

Analysis of Pollen Tetrad Data

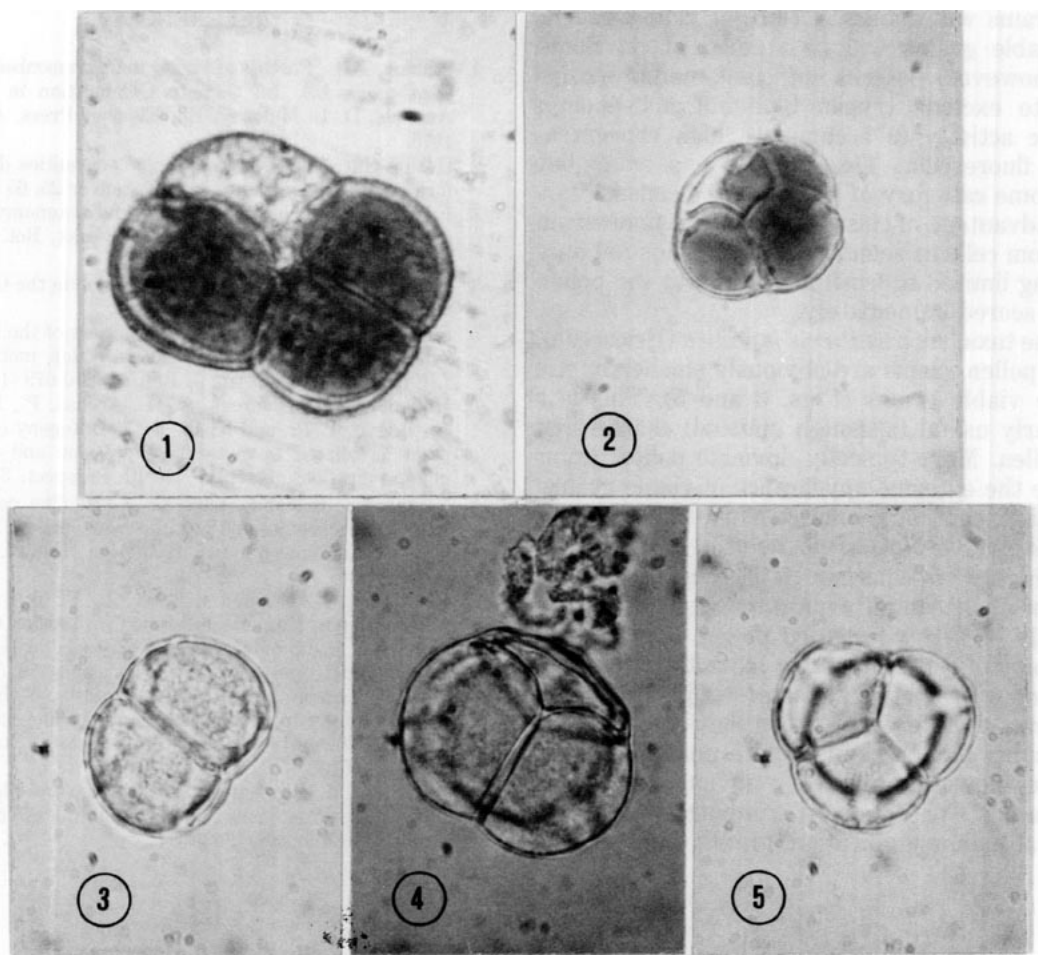
In calculating the incidence of environmental mutagenesis, it is necessary to consider that a tetrad which contains a single inviable pollen grain will mark the occurrence of a single postpachytene mutation. Similarly, a tetrad which contains two inviable pollen grains will, in most cases, indicate that diploid cell which was ancestral to that tetrad had also undergone a single mutagenic event. In some, the frequency of which is easily calculated (Table 1), tetrads containing only two viable grains may result from two postpachytene mutations, each of which induces a single inviable pollen grain. Finally, tetrads which contain three inviable pollen grains may be the result of one prepachytene mutation and one postpachytene mutation or, conversely, the result of three separate postpachytene mutational events. Table 1 indicates the probabilities of these different events. Because segregation is here assumed to be an exclusive characteristic of environmentally induced mutations, it is not possible to deal with tetrads which contain four inviable pollen grains. They could be the result of series of mutational events or alternatively, the consequence of environmental stresses. Thus they are ignored in this method.

In Table 1, g denotes number of genes which influence pollen viability and which are capable of mutation, and m is the probability that any one of these genes will mutate either before or after meiosis. According to these assumptions, the number of tetrads containing three viable pollen grains should be almost exactly twice the number of tetrads which contain only two viable grains. However, a survey of tetrads in *Pieris japonica* indicated that among 2057 tetrads which contained at

least one viable pollen grain, 57 (=2.77%) contained only three viable grains whereas 251 (=12.22%) contained only two viable grains. Similarly, among 4084 such tetrads of *Kalmia latifolia*, 153 (=3.74%) and 343 (=8.39%) contained only three and two viable grains, respectively, these data indicate that the probability of prepachytene mutations in Kalmia is 4.48 times, and in Pieris, 8.82 times, that of postpachytene mutations. These figures are close to those assumed by Hodgdon (*6*) and they point to a major difficulty of using data from individual pollen grains (*7*). Distinguishing between a single early mutation and an episode of multiple late mutations possess substantial difficulties in many tests for mutagenesis. Mutations which occur just prior to pachytene will each give rise to a single tetrad which contains only two viable pollen grains, but earlier in development, a single mutation many give rise to a large sector of tetrads each of which bears only two viable pollen grains. However, if pollen in tetrads is examined, the problem of mutant sectors can be avoided by recording only the frequency of tetrad which contain only three viable pollen grains. Each such tetrad represents a single postpachytene mutation.

Tetrad Methodology in Selected Taxa

In a number of species, the time honored test for pollen viability is aniline blue (0.05%) dissolved in lactophenol (*8*). This method is especially useful because it is simply a test for the presence of cytoplasm within pollen grains and thus it may be applied to preserved pollen, including that from herbarium specimens. Its use is illustrated in Figure 1, which shows to tetrad of *Typha latifolia*



FIGURES 1–5. Pollen tetrad from (1) *Typha latifolia*, (2) *Kalmia latifolia* and (3–5) *Pieris japonica*. See text for explanation.

(Typhaceae) pollen, and in Figure 2, which illustrates a tetrad of pollen from *Kalmia latifolia* (Ericaceae). Certainly the differences between filled (and presumably viable) and unfilled (inviable) grains is clear. As an arbitrary indicator of pollen viability, the method has the virtue of great simplicity. However, it is hardly to be supposed that all pollen grains which contain cytoplasm are viable. Thus the aniline blue method must overestimate pollen viability and thus underestimate mutagenic events.

Ockendon and Gates (*9*) have reported that the fluorescein diacetate test of pollen viability, developed by the Heslop-Harrison's (*10*), is more accurate than aniline blue as a predictor of pollen germinability, although, as a true test of metabolic activity, this method requires fresh pollen.

The fluorescein diacetate test may be improved upon by a simultaneous counterstain of trypan blue (0.25%) (*11*). If a suspension of pollen is stained with this mixture and then illuminated which ultraviolet light and a weak background of visible light, viable grains will fluoresce a bright yellow-green, and inviable grains will be stained black. Some grains, however, possess sufficient membrane integrity to exclude trypan blue but not enough metabolic activity to accumulate high concentrations of fluorescein. This results in a small but troublesome category of intermediate cells. A further disadvantage of this method is that fluorescein is lost from cells as soon as they die. Thus not only is staining limited to fresh material, but the pollen must be scored immediately.

In some taxa, such as *Pieris japonica* (Ericaceae), inviable pollen grains are obviously smaller in size than are viable grains (Figs. 3 and 5). This is a particularly useful (although unusual) characteristic of pollen. More typically, inviable pollen grains will have the external appearance of viable grains. Given this exception, a solution of 5-10% sucrose, or alternatively, 8*M* NaOH, can be used to render the tetrads highly transparent. It is then possible to determine their contents quite unambiguously.

A study of *Pieris japonica* pollen tetrads illustrates some of the contrasting advantages of volumetric and colorimetric indicators of pollen viability. Volumetric differences, when they are available, can certainly be determined quite unambiguously. They are, however, not very obvious under low magnification. Consequently, scanning must be carried out at high magnification, greatly reducing the speed with which tetrads can be scored. Colorimetric indications of pollen viability are sometimes rather ambiguous but suspected cases of inviable grains can be detected in a visual field of low magnification. These considerations suggest that an investigator equipped with only standard microscopes would be well advised to use a species which gives a volumetric indication of pollen viability. With a flow cytometer to make fluorescent measurements on large numbers of tetrads, fluorescent and visible light techniques might be the method of choice. However, unless unambiguous colorimetric methods are available, it may be that the best method would be to employ a Coulter counter, or other instruments which determine volume of small particles. Such counters are widely available and have the added advantage that they could be used to score both fresh and preserved material.

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